Appendix 2

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Insect cells as hosts for the expression of recombinant glycoproteins

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Baculovirus-mediated expression in insect cells has become well-established for the production of recombinant glycoproteins. Its frequent use arises from the relative ease and speed with which a heterologous protein can be expressed on the laboratory scale and the high chance of obtaining a biologically active protein. In addition to *Spodoptera frugiperda* Sf9 cells, which are probably the most widely used insect cell line, other mainly lepidopteran cell lines are exploited for protein expression. Recombinant baculovirus is the usual vector for the expression of foreign genes but stable transfection of - especially dipteran - insect cells presents an interesting alternative. Insect cells can be grown on serum free media which is an advantage in terms of costs as well as of biosafety. For large scale culture, conditions have been developed which meet the special requirements of insect cells.

With regard to protein folding and post-translational processing, insect cells are second only to mammalian cell lines. Evidence is presented that many processing events known in mammalian systems do also occur in insects. In this review, emphasis is laid, however, on protein glycosylation, particularly N-glycosylation, which in insects differs in many respects from that in mammals. For instance, truncated oligosaccharides containing just three or even only two mannose residues and sometimes fucose have been found on expressed proteins.

These small structures can be explained by post-synthetic trimming reactions. Indeed, cell lines having a low level of *N*-acetyl-β-glucosaminidase, e.g. *Estigmene acrea* cells, produce N- glycans with non-reducing terminal N-acetylglucosamine residues. The *Trichoplusia ni* cell line TN-5B1-4 was even found to produce small amounts of galactose terminated N-glycans. However, there appears to be no significant sialylation of N-glycans in insect cells. Insect cells expressed glycoproteins may, though, be α1,3-fucosylated on the reducing-terminal GlcNAc residue. This type of fucosylation renders the N-glycans on one hand resistant to hydrolysis with PNGase F and on the other immunogenic. Even in the absence of α1,3-fucosylation, the truncated N-glycans of glycoproteins produced in insect cells constitute a barrier to their use as therapeutics. Attempts and strategies to "mammalianise" the N-glycosylation capacity of insect cells are discussed.

Keywords: **insect cells, baculovirus, N-glycans, insect glycoproteins**

Abbreviations: **GPI, glycosylphosphatidylinositol; MGn, abbreviation for N-glycan, see Figures 2 and 5; MOI, multiplicity of infection; PNGase, peptide-*N*^{4}-(*N*-acetyl-β-glucosaminyl)asparagine amidase.**

Introduction

The reasons why one may want to express a recombinant protein are as diverse as the proteins themselves. The high end of the spectrum undoubtedly is the production of human therapeutic proteins which pose extreme demands in terms of product quality and safety but also offer the advantage of high product prices. We shall, however, not forget that there are many other applications of recombinant technology having lower demands for product quality (e.g., the production of veterinary therapeutics and diagnostics). Most recombinant expressions are however made for strictly scientific purposes such as the verification of the identy of a protein or cloned gene, or studies on the function and structure of a protein.

A variety of expression systems have been developed which are currently subject to a process akin to a biological evolution and selection where, however, improvements to a system are not merely caused by chance. The expression systems will occupy different 'ecological' (i.e., biotechnological) niches, some will thrive and yet others may go extinct. The authors strongly believe that insect cells as expression systems for foreign proteins will stand their ground. This notion is, for instance, exemplified by an incidental observation: seven out of eight recombinant glyco-

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syltransferases currently offered by Calbiochem are produced in insect cells. Numerous experiments are conducted with insect cell expressed proteins and it is impossible to include all these references in this review. However, we shall emphasise the usefulness of the insect cell baculovirus system for the production of dimeric or multimeric proteins as e.g. IgG [1,2], complement factors [3]; viral antigens [4–6] and even heteropentameric proteins [7,8]. Other interesting applications are functional studies of mammalian receptor proteins using transformed insect cells [e.g., 9–13] or the use of recombinant baculovirus for an epitope expression library [14].

In this review we will start with a broad view on many aspects of protein expression in insect cells with reference to more detailed descriptions. We will focus on the issue of posttranslational modifications, especially on protein N-glycosylation in insects. The particularly interested reader is also referred to recently published reviews dealing with N-glycosylation in insects and insect cells [15–17].

Biotechnological aspects of insect cell expression systems

Cell lines and organisms

Excepting some work with *Drosophila melanogaster* cells (most often S2 or Kc cells) and mosquito cells [10,18], most heterologous expressions have been and are performed with lepidopteran cells. Within these, the closely releated Sf9 and Sf21 cells from the fall army worm *Spodoptera frugiperda* are by far the most frequently used cell lines. Indeed, the term "insect cells" has almost become a synonym for Sf9 / Sf21 cells. However, quite a number of other cell lines has been established (see e.g., [19]). Interesting cell lines stem from *Trichoplusia ni,* i.e., TN-368 and BTI-TN-5B1-4; the latter being commercially available as "High-Five™" and claimed to give especially high yields of recombinant protein, *Bombyx mori* (Bm-N), *Mamestra brassicae* (e.g., MB0503), and *Estigmene acrea,* which is notable mainly because of its glycosylation potential (see below). In general, stable cell lines are usually obtained from embryonic cells and thus represent essentially undifferentiated cells.

An alternative strategy to the use of insect cell culture is to infect whole larvae with recombinant baculovirus (e.g., [2,20,21]). However, there appear to be few data on the thorough structural characterisation of recombinant proteins expressed in larvae. Only in a recent report, the first of its kind to the authors knowledge, the N-glycans of recombinant secreted alkaline phosphatase expressed in a variety of insect larvae have been investigated [22].

Vectors

The most widely used vectors for the production of foreign proteins in insect cells or larvae are recombinant baculoviruses, such as *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) which infects lepidopteran cells [23,24]. In special cases, the *Bombyx mori* nuclear polyhedrosis virus (BmNPV) is used [2,20,22]. While the construction of baculoviral vectors is considered to be relatively convenient, much effort is made to reduce further the time required and to improve the yield of recombinants [25–28]. Foreign proteins are expressed under the control of the viral polyhedrin promoter which is an especially strong promoter responsible for the particularly high protein expression which usually by far surpasses the productivity obtained with mammalian *in vitro* expression systems. Therefore purification of the recombinant protein is relatively simple and may be even further facilitated by expressing a fusion protein with a purification tag such as polyhistidine [29–31].

However, baculoviruses essentially have a lytic infection mode, i.e. when product is harvested, a large proportion of the host cells are lysed and releases degradative enzymes. In addition, the protein biosynthesis is maximal near death of infected cells and it is possible that the overall processing of the protein is suboptimal at that time—particularly proteins destined for the plasma membrane or for secretion are affected by the depletion of components of the posttranslational machinery of the secretory pathway.

Attempts to circumvent this problem include the use of early baculovirus promoters in either transiently or stably transformed cells [10,18,27,32]. Much of this work was done with *Drosophila melanogaster* or mosquito in addition to *Spodoptera* cells. The rate of protein expression in stably transformed cells will often—but not necessarily—be significantly lower than with the conventional baculoviral system [18]. For certain purposes this might even be an advantage, for instance in functional studies of recombinant receptor proteins [18,27,32]. A recent trend is the use of early expression vectors and/or stably transformed lepidopteran cells to improve protein folding or N-glycosylation in insect cells as will be discussed below [33–39].

Growth media and production processes

An especially attractive feature of the insect cell system is the possible use of serum free and even protein free growth media. This confers benefits in terms of production costs and biosafety (see also below) [40–45]. On the negative side, large scale insect cell culture offers particular challenges to the biotechnologist due to the higher oxygen consumption and higher shear sensitivity as compared to mammalian cells [46]. Several ways have been shown to successfully cope with these problems by appropriate bioreactor design. Interestingly, very different systems such as attached cell culture (mainly with perfusion reactors) or suspension culture with either airlift reactors or stirred tank reactors have been used successfully [40,42,47–50].

Insect cells in suspension culture can be protected from shear stress by the addition of detergents, mostly Pluronic F-68 (Synperonic F68) [40,43]. High-Five™cells are attachment-dependent and require special conditions [51].

The baculovirus insect cell system constitutes an essentially discontinuous batch-wise production system. Normally, insect cells are grown to a density optimal for efficient baculovirus infection and recombinant baculovirus is added at an appropriate multiplicity of infection (MOI). Unfortunately, since high cell densities for unknown reasons exert a negative effect on infection efficiency, this density will usually lie well below the maximal possible cell density. Usually a MOI of 2–5 is chosen, but MOIs ranging from 0.01 to 50 have led to good expressions of recombinant proteins. There seems to be a close interrelation between several factors such as cell line, medium, cell density (or time of infection), MOI and final protein yield and quality so that sometimes different conditions may lead to similar results.

Recombinant protein, very often in a secreted form, is harvested after several, typically four to five days post infection and then subjected to some sort of downstream processing [52]. It has to be kept in mind that at that time a considerable fraction of the cells has already lysed and thus the protein of interest is exposed to a number of degradative enzymes [53]. The time of maximal yield therefore is not necessarily the optimal time of harvest.

Safety and regulatory aspects

As intimated above, the ability of insect cells to grow on media devoid of foetal calf serum or other components of animal origin, reduces risks introduced by such media additives. Indeed the possible contamination of calf serum with bovine spongiform encephalopathy (BSE) prions is an issue of concern. It may also be speculated that cells derived from organisms which are phylogenetically very distant to humans, will generally less likely be hosts for human pathogenic viruses than mammalian cells. However, this notion maybe somewhat naive, since some pathogenic arboviruses causing for example, yellow fever or Dengue fever are able to replicate in insects [45].

Any new human therapeutic has to be approved by a relevant legal authority, such as the U.S. food and drug administration (FDA). To the best of the authors' knowledge, no insect cell derived recombinant protein has hitherto been subjected to an official approval process. However, as more and more proteins are produced in insect cells in the preclinical stage, there is some attractiveness in the idea to ride this horse right to the market. Particularly promising is the field of viral vaccines where the formation of virus-mimicking multimers can be essential for eliciting a protective immune response. Thus, a hepatitis C vaccine might well be the first human therapeutic expressed in insect cells [6].

Posttranslational events and protein folding

Generally, with respect to the ability to produce fully processed and biologically active recombinant proteins, insect cells are second only to mammalian cells. The foreign DNA codes for a certain peptide sequence, and with just a little bit of luck, the primary structure of the newly translated polypeptide will be exactly the same whether it is expressed in insect cells, in mammalian cells or in the original organism. However, in most cases this primary product is subject to a range of processing events before it attains its final structure. The fundamental maturation step is protein folding including formation of disulphide bonds. Folding can be accompanied or followed by quite a diverse array of reactions summarised under the term posttranslational modifications. Proteins destined for different intracellular compartments are subject to very different posttranslational modifications. For example, the addition of O-GlcNAc is believed to happen only with nuclear and cytosolic proteins while other glycosylation reactions happen along the endomembrane assembly line and thus are relevant only for secretory, lysosomal and membrane-bound proteins. However, the situation is not that simple as membrane proteins e.g. herpesvirus glycoprotein B when expressed in insect cells, may be targeted to the nuclear membrane [54].

There is evidence that insect cells can perform most of the processing steps occurring in mammalian cells. As a consequence, there is a greater probability that a protein expressed in insect cells will have normal biological activity than is the case with a protein expressed in *E. coli* (e.g., [55–57]). However, when compared to the authentic protein by SDS-PAGE, the insect cell derived protein very often exhibits a slightly lower molecular mass. Three major reasons for such an effect are differences in (1) proteolytic processing, (2) glycosylation site occupancy, and (3) glycan structure. The effect of such differences on thermal stability and—possibly as a result—on the enzymatic activity of the recombinant protein may vary from case to case and is often moderate. However, for therapeutic applications altered circulation half life and the possible presentation of immunogenic determinants are serious issues.

Different insect cell lines vary in their potential for proteolytic processing but it seems that in principle the responsible proteases, in particular those for signal peptide cleavage, are similar to those acting in mammalian cells [58]. N-Glycosylation at the well known tripeptide sequence Asn-X-Ser/Thr occurs in insect cells. While in principle the same sites are glycosylated by mammalian and by insect cells [59,60], under-glycosylation is sometimes observed (e.g., [61,62]). The structures of N-glycans found on insect cell expressed glycoproteins will be discussed in a later section.

Much less is known about the use of O-glycosylation sites in insect cells. Human interferon-α2 expressed in Sf9

cells was O-glycosylated at the same position as the natural interferon [63]. However, in contrast to the structurally diverse, sialylated mammalian O-glycans, those found on insect glycoproteins consisted of either GalNAc alone or GalNAc and Gal [63–67]. In the case of human interleukin 2 expressed in Sf 21 cells, the structure of its O-glycan was determined to be the core 1 disaccharide Galβ1-3GalNAc, also known as Tn-antigen [67]. By lectin binding this epitope was also detected in *Drosophila melanogaster* mucin [68]. Binding of *Bandeiraea simplicifolia* lectin-I isolectin B_4 to extracts of a *M. brassicae* cell line indicated the presence of Galα1,3/4Gal sequences [69]. Indeed, the cells contained an α1,4-galactosyltransferase which used Galβ1-3GalNAcα1-O-benzoyl but not asialofetuin as an acceptor [69]. Cytosolic and nucleoplasmic proteins of many eukaryotic organisms are known to contain O-linked GlcNAc residues. Kelly & Hart [70] showed *Drosophila* polytene chromosomes to contain O-GlcNAc residues. Human keratins 8 and 18 expressed in Sf9 cells contained O-GlcNAc (as well as phosphate) in positions very similar to the natural keratins [71].

Certain membrane proteins are "anchored" in the lipid bilayer by means of a glycosylphosphatidylinositol anchor (GPI anchor). Part of the human CD59 antigen expressed in *Spodoptera frugiperda* cells appeared to carry a GPI anchor as judged from phase partition experiments [72]. However, no structural details have been given so far for an insect GPI anchor. Acyl residues may also be found in direct linkage to the polypeptide. There is good evidence that *Spodoptera frugiperda* cells can furnish proteins with cysteine-linked and hence hydroxylamine-sensitive palmitoyl residues [73–78]. The palmitoylation of virus proteins by mosquito (*Aedes albopictus*) cells must be mentioned here [79]. However, as judged from saponification experiments, fatty acid residues may also occur in oxyester linkage [76,80].

Several other posttranslational modifications are known and it is held that insect cells are capable of performing phosphorylation of serine residues [9,71,81,82], tyrosine sulfation [81], acetylation of N-terminal serine residues [82], and, according to a recent review, also C-terminal amidation and β-hydroxylation of aspartate residues [3]. When the prolactin receptor was expressed in Sf9 cells, the product was larger than could by explained by its sequence and by glycosylation. Western-blots revealed the receptor to be ubiquitinylated on its cytoplasmic side [83].

In conclusion, insect cells have been shown to perform most, if not all, of the posttranslational modifications known to occur in mammalian cells. However, expression of a given protein in a certain cell line does not necessarily result in a product identical to the authentic protein. Even if the protein is modified in a certain way the processing may be incomplete and hence a heterogeneous product is obtained. The separation of isoforms and the thorough structural comparison of natural and recombinant proteins therefore presents an extremely difficult and demanding task. Fortunately, for many purposes, a product which is functional within a limited context, e.g. for studies on enzymatic activity, binding specificity, receptor function etc., is sufficient.

A prerequisite to the production of high amounts of active protein is the correct folding of the newly synthesised polypeptide. Very often, this is only possible in the presence of suitable chaperones. A given insect cell may not provide a sufficient level of the required chaperoning activity thus leading to protein denaturation and aggregation and consequently poor product yield. Therefore, the co-expression of specific chaperones may significantly enhance the yield of active and secreted protein. In the case of immunoglobulins, immunoglobulin heavy chain binding protein (BiP) was found to improve the systems productivity [33–36]. In another study, co-expression of a cyclophilin which catalyses the *cis/trans* isomerisation about Xaa-Pro bonds directly or indirectly led to the improved folding of a human transporter protein [37].

N-glycosylation in insect cells

Besides protein folding, generation of disulphide linkages and proteolysis, the attachment of asparagine-linked oligosaccharides is the most prominent posttranslational event of protein biosynthesis. While the enzymatic function of some proteins, such as bovine pancreatic ribonuclease, bee venom phospholipase A_2 [84] or, to mention a heterologous protein, a potassium channel [85] is not detectably affected by the presence or absence of N-linked glycans, many proteins will not attain full (enzymatic) activity if expressed in an un- or underglycosylated form (e.g., [56]). In contrast, the exact structure of the N-glycans will usually play a marginal role (e.g., [86]). Generally, the carbohydrate moiety is to be regarded as having significant effects on both the structure and on the physicochemical features of a protein and thereby its enzymatic activity, antigenicity and (thermal) stability [87]. The large hydrophilic glycan may already play a vital role during protein folding [88]. Moreover, chaperoning of glycoproteins in the endoplasmic reticulum is partly mediated by their N-glycans. Misfolded proteins are recognised and re-glucosylated by UDP-Glc:glycoprotein glucosyltransferase. The mono-glucosylated N-glycan then serves as a recognition signal for the lectin-like chaperones calnexin and calreticulin [89,90]. While N-glycosylation may be vital during protein folding it might be unfavourable for the mature protein and this could explain the finding of transient glycosylation in *Drosophila* rhodopsin [91].

Apparently the reglucosylation cycle also exists in insects [18,92] and there is evidence for the expression of α-glucosidases I and II [93]. An essential ingredient of this quality control mechanism is the presence of oligomannosidic N-glycans on the newly synthesised protein. Indeed, already in 1984, the presence in mosquito cells and later in *Drosophila* of the lipid-linked precursor oligosaccharide

$Glc_3Man_9GlcNAc_2$ was reported [94,95] and in 1985, Ryan and his co-workers presented the analysis by NMR of $Man_9GlcNAc_2$ from a *Manduca sexta* glycoprotein [96]. From these data it became clear that the early steps of protein N-glycosylation in insects resemble those in mammals and, as we know today, in essentially all eukaryotes. The presence of oligomannosidic N-glycans with five to nine mannose residues (Man5, Man6, Man7, Man8 and Man9) has since been reported many times for endogenous insect glycoproteins as well as for recombinant glycoproteins expressed in insect cells [60,97–105]. Where analysed, the major or only detectable Man8 isomer was Man8(1,3) (Fig. 1) accompanied by traces of Man8(1,2), whereas roughly comparable amounts of Man7(1) and Man7(3) have mostly been found [60,99,100,104].

Remarkably, the multiply glycosylated proteins HIV gp120 and bovine lactoferrin when expressed with the baculovirus system carried oligomannosidic N-glycans on the same sites as did their mammalian counterparts (recombinant in CHO cells and natural, respectively) [59,60]. The other glycosylation sites bear complex N-glycans. However, with insect glycoproteins, this term has a significantly different meaning than with mammalian glycoproteins as will be shown in the following section.

Complex N-glycans

In mammalian glycoproteins the majority of N-glycans are of the complex type, i.e. they consist of a pentasaccharide core of two GlcNAc and three mannose residues with antennae comprising of additional GlcNAc, galactose, sialic acid and often fucose residues. An enormous diversity of impressively complex oligosaccharide structures is thereby possible. We shall not discuss in detail the possible biological functions of complex N-glycans. Recent experiments with knock-out mice unable to synthesise complex N-glycans clearly point out their essential role—and what a role! The mice die before being even born [106,107].

In contrast to the wonderfully varied multiantennary, sialylated complex N-glycans produced in mammalian cells, their poor relatives on insect cell expressed glycoproteins can hardly be called "complex" and the terms truncated, paucimannosidic or modified appear to be more justified for these N-glycans. In quite a number of cases the "complex" N-glycans attached to either recombinant, endogenous or viral glycoproteins consisted of just the common pentasaccharide core—sometimes even lacking the α1,3-linked mannosyl residue—with or without fucose linked α 1,6 to the proximal GlcNAc residue (Fig. 2) [22,60,61,65-67,98–100,102–104,108–115]. Most of these studies were performed with material obtained from *Spodoptera* cells, but the same structures have also been found on glycoproteins produced by cells or larvae from *Drosophila melanogaster* [99], *Mamestra brassicae* [60,100], *Bombyx mori* [22,100], *Trichoplusia ni* [104,114,115], *Estigmene acrea* [102,103,115] and other organisms [22]. So much about the widespread occurrence of the "boring four" depicted in Figure 2. However, depending on the host cell and, as we think, to some degree on the analytical methodology used, additional substituents may be found.

α1,3-fucosylated core GlcNAc

Substitution by fucose of the C3 hydroxyl group of the Asn-linked GlcNAc was originally discovered in the plant glycoprotein bromelain [116]. This work was pioneering in yet another way since this is the first report on the use of a peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase (PNGase) to cleave glycan from peptide. This enzyme was purified from almond hence its name PNGase A or almond glycopeptidase. In contrast to the now more widely used PNGase F from *Flavobacterium meningosepticum*, the almond glycopeptidase is capable of hydrolysing N-glycans with an α1,3-fucosylated core GlcNAc [117,118] and was used in the authors' laboratory to release glycans from insect glycoproteins. Indeed, the structural analysis of the N-glycans from honeybee venom phospholipase A_2 revealed that in insect glycoproteins the proximal GlcNAc residue can be fucosylated either at position 6, as is well known from mammalian glycoproteins, as well as in position 3 as was until then believed to occur only on plant glycoproteins [119,120]. The corresponding α1,3-fucosyltransferase activity could be detected in both honeybee venom glands and *M. brassicae* cells [121,122]. The enzymes from both sources were able to act on non-fucosylated as well as on 6-fucosylated acceptors thereby generating difucosylated N-glycans [123]. Indeed, significant amounts of α1,3-and difucosylated structures (Fig. 3) were found on membrane glycoproteins from *M. brassicae* cells, but also from *S. frugiperda* and *B. mori* cells [100]. The *T. ni* cell line BTI-TN-5B1-4 evidently does α1,3-fucosylate recombinant proteins as 18% of the N-glycans of recombinant IgG secreted by these cells were difucosylated (Fig. 4) [104]. Interleukin-2 produced in both BTI-TN-5B1-4 and IZD MB0503 contained glycans with 2 to 3 Man and 1 to 2 Fuc residues and hence at least these two cell lines appear to have a significant ability to α1,3-fucosylate the Asn-bound GlcNAc of recombinant proteins [115]. However, when human interferon ω1 expressed in Sf9 cells was analysed no α1,3-fucosylated N-glycans could be detected even though the authors used PNGase A to liberate the oligosaccharides [61]. Since Sf9 cells display a low capacity for 3-fucosylation [100,122], it is feasible that recombinant glycoproteins are not or only marginally α1,3-fucosylated potentially due to a depletion of the required transferase as a consequence of baculovirus infection—a phenomenon which has been observed for GalNAc-transferase in *T. ni* cells [124]. Regrettably, this question remains open since in many studies, PNGase F has been used to liberate the oligosaccharides and so the results of these studies are

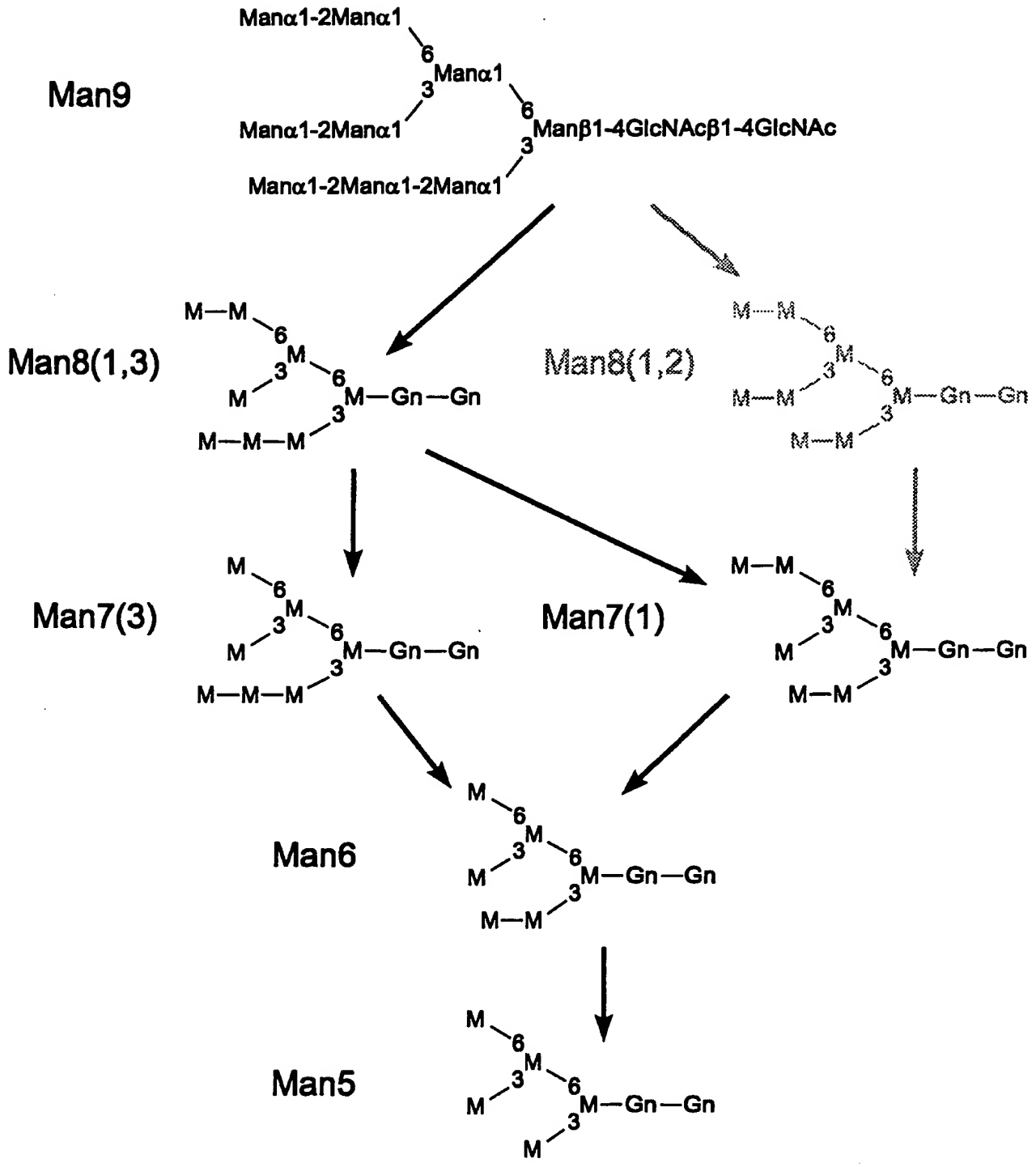

Figure 1. Oligomannosidic N-glycans from glycoproteins expressed in lepidopteran insect cells. The trimming pathway is deduced from oligosaccharide structures (described in Refs. [97,101]). In contrast to Man9, Man6 and Man5, the N-glycans Man8 and Man7 come in different flavours. Three isomeric structures are possible for both Man8 and Man7. A simple abbreviation system is proposed for these isomers: The structure is read from top left downwards. On the major Man8 isomer, α-mannosidase has only acted on the second antenna, and not the first and on the third antenna, hence the abbreviation Man8(1,3).

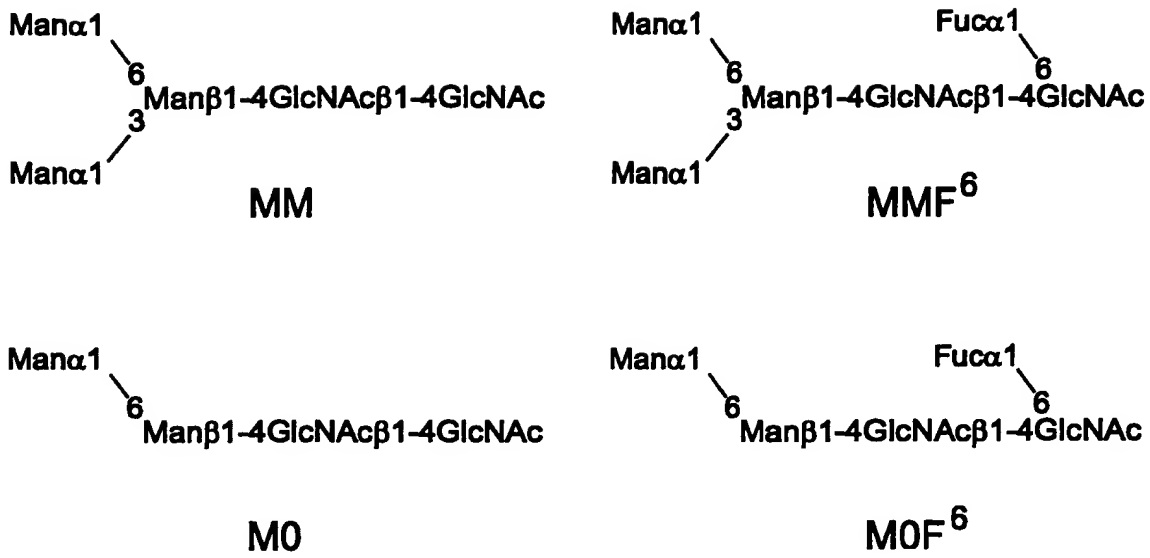

Figure 2. The "boring four": frequently-found truncated N-glycans from glycoproteins expressed in lepidopteran insect cells. Especially the structures with three mannose residue have been found on almost any insect derived glycoprotein (see text). Because of their low number of mannose residue they are sometimes referred to as "paucimannosidic" N-glycans. The abbreviation system (derived from that of Dr. Harry Schachter) at first denotes the terminal residue on the upper and then on the lower antenna and finally substituents of the core are listed. Mannose residues are represented by M, GlcNAc residues by Gn (see Fig. 5) and fucose residues by an F. However, since fucose may also occur in α1,3-linkage (see Fig. 3), its linkage is specified by superscripts. It shall be noted that the four structures shown in this figure are sensitive to both PNGase F and A while MM and MMF^6 are also sensitive to endo-β-*N*-acetylglucosaminidase D [125].

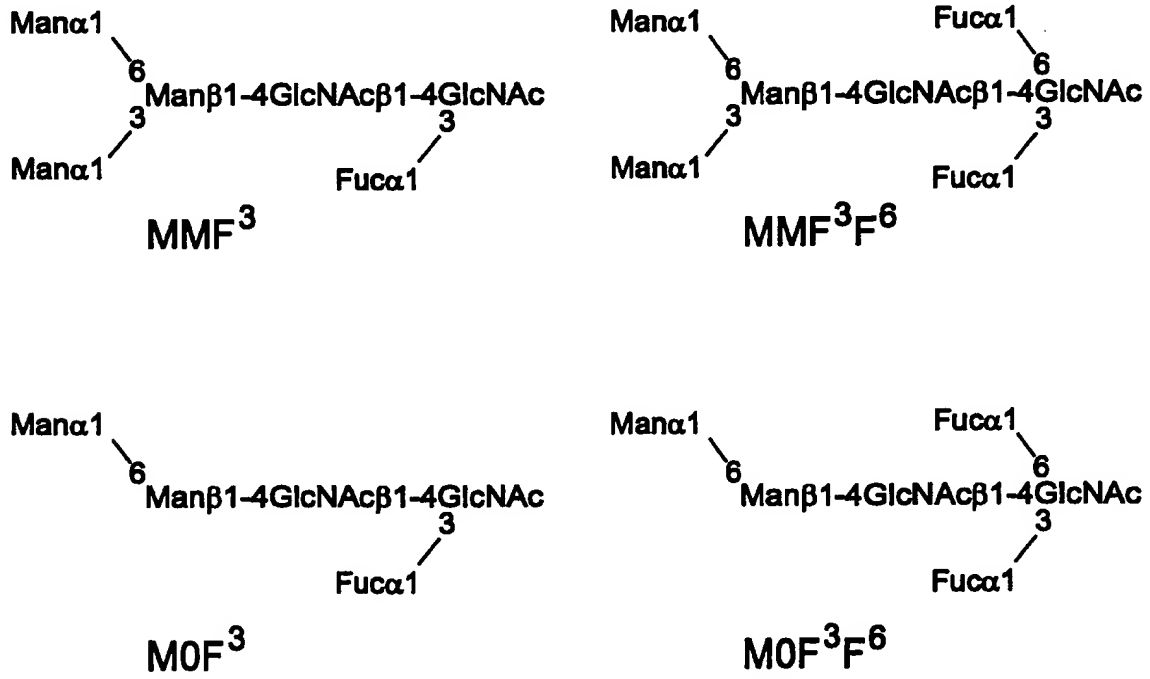

Figure 3. α1,3-Fucosylated and difucosylated insect N-glycans. Such structures have been detected in honeybee venom phospholipase A_2 (14 and 7%) and hyaluronidase (35 and 46%) and on membrane proteins from *Spodoptera frugiperda* (2 and 9%), *Mamestra brassicae* (5 and 26%) and *Bombyx mori* (<1 and 3%) cells [100]. The numbers in brackets give the relative amounts of core α1,3-fucosylated and difucosylated N-glycans, respectively. These structures cannot be liberated by PNGase F or endo-β-*N*-acetylglucosaminidase D [117,118,125]. The abbreviation system is explained in the legend to Figure 2.

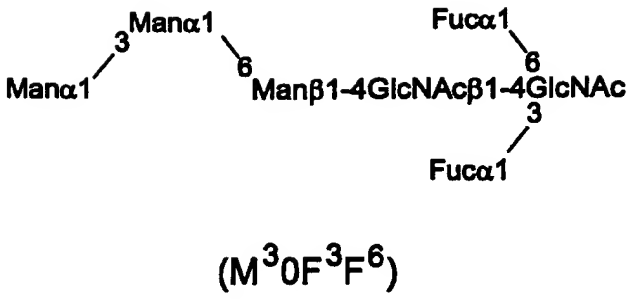

Figure 4. Difucosylated N-glycan found in IgG expressed in *Trichoplusia ni* cells [104].

necessarily a bit incomplete. Indeed, alternative release methods are problematic either. Endoglycosidase D is also inhibited by core α1,3-fucosylation [125], therefore, the resistance of insect N-glycans towards endoglycosidases D and H must not be interpreted according to the rules for mammalian glycoproteins. Hydrazinolysis likewise does not appear to be a good choice for insect N-glycans since a substituent at position 3 of the Asn-bound GlcNAc is prone to elimination [126,127].

The occurrence of core 3-fucosylation is not of purely academic interest; it is positively undersirable in the case of therapeutic glycoproteins as α1,3-fucosylation renders the N-glycan immunogenic to mammals [128–130]. α1,3-Fucosylated oligosaccharides occur on innumerable plant glycoproteins [131] and essentially represent the "cross-reactive carbohydrate determinant" (CCD) which is common not only to almost all plants, but also to insect glycoproteins [132]. Antiserum raised in rabbits against horseradish peroxidase contains a significant fraction of antibodies directed against α1,3-fucosylated N-glycans [128,129,133]. Remarkably, such antisera are used to stain neuronal cells in insect embryos [134–137]. While this could be a starting point for nice speculations we content ourselves with pointing out that natural insect tissues display highly differential α1,3-fucosylation.

Terminal GlcNAc and the biosynthesis pathway

The presence of N-glycans of the structures depicted in Figures 2 to 4 raises questions regarding their biosynthesis. In mammals, all the enzymes acting beyond the oligomannosidic structure $Man_5GlcNAc_2$ (M5) require the presence of a GlcNAc-residue on the α1,3-linked mannose which is transferred by GlcNAc-transferase I [138]. Lepidopteran insect cells likewise contain significant amounts of this key enzyme for the processing of N-glycans [139,140]. In addition, α-mannosidase II and both fucosyltransferases acting on the proximal GlcNAc rely on the presence of non-reducing terminal GlcNAc [123,139,141,142]. Certainly the levels of α-mannosidase II must be significant in all insect cells studied so far, since no glycans with 5 mannose and additional GlcNAc or Fuc residues have ever been found. Low amounts of N-glycans with a mannose residue in α1,3-linkage to the α1,6-linked mannose of the core have been detected (e.g., Fig. 4) [104,120] and these structures are in accordance with the substrate specificity of insect cell α-mannosidase II [141,142].

The missing link between the said substrate requirements and the presence of structures lacking terminal GlcNAc as depicted in Figures 2 to 4 appears to be an *N*-acetyl-β-glucosaminidase (GlcNAcase) acting at some point along the endomembrane assembly line. Indeed, a membrane-bound GlcNAcase can be found in Sf21, Bm-N and MB0503 cells [143]. This "processing GlcNAcase" has a remarkable substrate specificity, i.e. it hydrolyses $GlcNAcMan_3GlcNAc_2$ but not $GlcNAcMan_5GlcNAc_2$ [143,144]. As a consequence, the N-glycans found on glycoproteins produced by these cell lines contained only very small amounts of terminal GlcNAc [100,144]. Remarkably, the single terminal GlcNAc was in part linked to the α1,6-mannose of the core (Fig. 5) indicating the action of

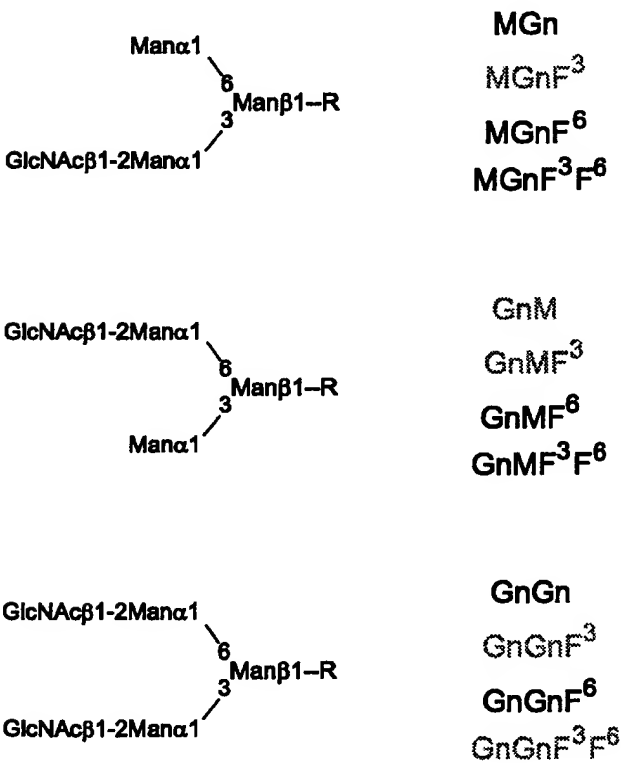

Figure 5. N-glycans with terminal GlcNAc-residues in insects. Such N-glycans may be present on glycoproteins expressed in any lepidopteran cell but especially in *Trichoplusia ni* and *Estigmene acrea* cells (see text). The fucosylation status of the core is not shown in the structural formulas and may vary as shown in Figures 2 and 3 and as indicated by the abbreviations. Only structures represented by abbreviations in bold print have hitherto been detected in insect glycoproteins.

GlcNAc-transferase II activity [100,139]. A cell line with a low GlcNAcase activity should produce larger amounts of these hybrid N-glycans. Indeed, haemagglutinin, interferon γ and β-trace protein when expressed in *E. acrea* Ea4 cells which have very little GlcNAcase, carried significant amounts of N-glycans with one, two or even three terminal GlcNAc residues [103,115,144]. The linkage of these GlcNAc residues has not yet been determined. Larger structures of the kind shown in Figure 5 also comprised a significant fraction of the N-glycans of recombinant IgG expressed in TN-5B1-4 cells [104].

In many cases, N-glycans without the core α1,3-mannose residue have been found. It is unknown whether the removal of this mannose residue is to be regarded as a biosynthetic event comparable to the action of the "processing GlcNAcase" or just as a manifestation of protein "ageing". Taken together, the processing of N-glycans in insect cells appears to follow the routes depicted in Figures 6 and 7. When we consider that MGn (as in Fig. 7) is just one possible starting substrate for fucosylation it becomes obvious that quite a number of different N-glycan structures may be found on insect cell derived glycoproteins. The actual number of different structures detected is determined by three major variables: the protein, the cell line and the analysis methodology. Analyses employing two-dimensional HPLC mapping of fluorescently labelled sugars [100,101,104,120] generally appear to reveal more complex glycosylation patterns than other approaches.

Galactose, GalNAc and sialic acid

Remarkably, a certain proportion of the glycans of IgG expressed in *T. ni* cells was even found to be galactosylated to give the LacNAc unit Galβ1-4GlcNAc [104]. Mass spectrometric analysis indicated the presence of galactosylated N-glycans on interferon γ expressed in *E. acrea* cells [103]. In other studies, however, no indication for the presence of Gal on glycoproteins expressed in *T. ni* or *E. acrea* cells was found [114,115].

Sialylation has not been observed in any of the studies cited above. Lectins have sometimes been used e.g. for the characterisation of the N-glycans attached to a phosphatase recombinantly produced in *T. ni* cells [145]. In a later study employing electrophoretic analysis of fluorescently labeled glycans, the same group did not find sialylated or galactosylated structures [22]. While this discrepancy may have one out of several possible reasons, it addresses the problem of reliability inherent to lectin blot analysis of glycoprotein glycans. The probably strongest indication of the presence of sialic acid has been given by its gas chromatographic-mass spectrometric identification in *Drosophila* larvae, where it was found as α2,8-polysialic acid [146].

Some lepidopteran cells appear to have the ability to generate 'LacdiNAc units' [147] by virtue of a β1,4-GalNAc-transferase [124]. Although this sequence has never been detected in recombinant glycoproteins produced in lepidopteran cells, N-glycans with the terminal trisaccharide GalNAcβ1-4(Fucα1-3)GlcNAcβ1-2 have, however, been found in proteins from the venom of the honeybee (Fig. 8) [101,120]. It is noteworthy that the N-glycans from a hymenopteran insect otherwise resemble those of lepidopteran and dipteran species (see above). In contrast, the orthoptera *Locusta migratoria* was found to produce unusual N-glycans with 2-aminoethylphosphonate linked to Man or terminal GlcNAc [148].

Lysosomal enzymes

In mammals, the oligomannosidic N-glycans of lysosomal enzymes become phosphorylated and the mannose 6-phosphate then serves as sorting signal during vesicular transport [149]. However, prorenin when expressed in *S. frugiperda* cells, did not contain phosphomannosyl glycans but rather the typical truncated N-glycans [109]. This result was also obtained for recombinant human arylsulphatase A (Schwihla H, Bachinger T, Altmann F, Glößl J, unpublished results).

Molecular biology of N-glycan biosynthesis in insects

Surprisingly few of the genes involved in the biosynthesis of N-glycans have been identified in lepidopteran or other insect genomes. To the authors' knowledge only genes encoding α-mannosidase I (or α1,2-mannosidase) and α-mannosidase II have been isolated so far and cloning was performed by homology to the respective mammalian enzymes in all cases. cDNAs encoding α1,2-mannosidase have been isolated from *D. melanogaster* [150] and from *S. frugiperda* [151]. Likewise, cDNAs for α-mannosidase II have been cloned from *D. melanogaster* [152] and from *S. frugiperda* [153]. The cloning of the UDP-Glc:glycoprotein glucosyltransferase responsible for reglucosylation of misfolded proteins (see above) should be mentioned again here [92]. While in these cases sufficient homology between insect and mammalian genes has been conserved, a cDNA probe for mouse GlcNAc transferase I did not detect a homologous gene in *Drosophila* [154].

Although not directly related to our topic, the purification and cloning of *S. frugiperda* glycosylasparaginase is noteworthy [155]. The recently cloned *N*-acetyl-β-glucosaminidase from *Manduca sexta* certainly is involved in the chitinolytic "moulting" of the insect cuticle but its relation, if any, to the processing GlcNAcase found in many lepidopteran cells has not been established [156].

Conclusion and perspectives

Is it possible to produce recombinant glycoprotein with mammalian-like N-glycans? Even though some insect cell lines (Ea4 or TN-5B1-4) exhibit a remarkable glycosylation

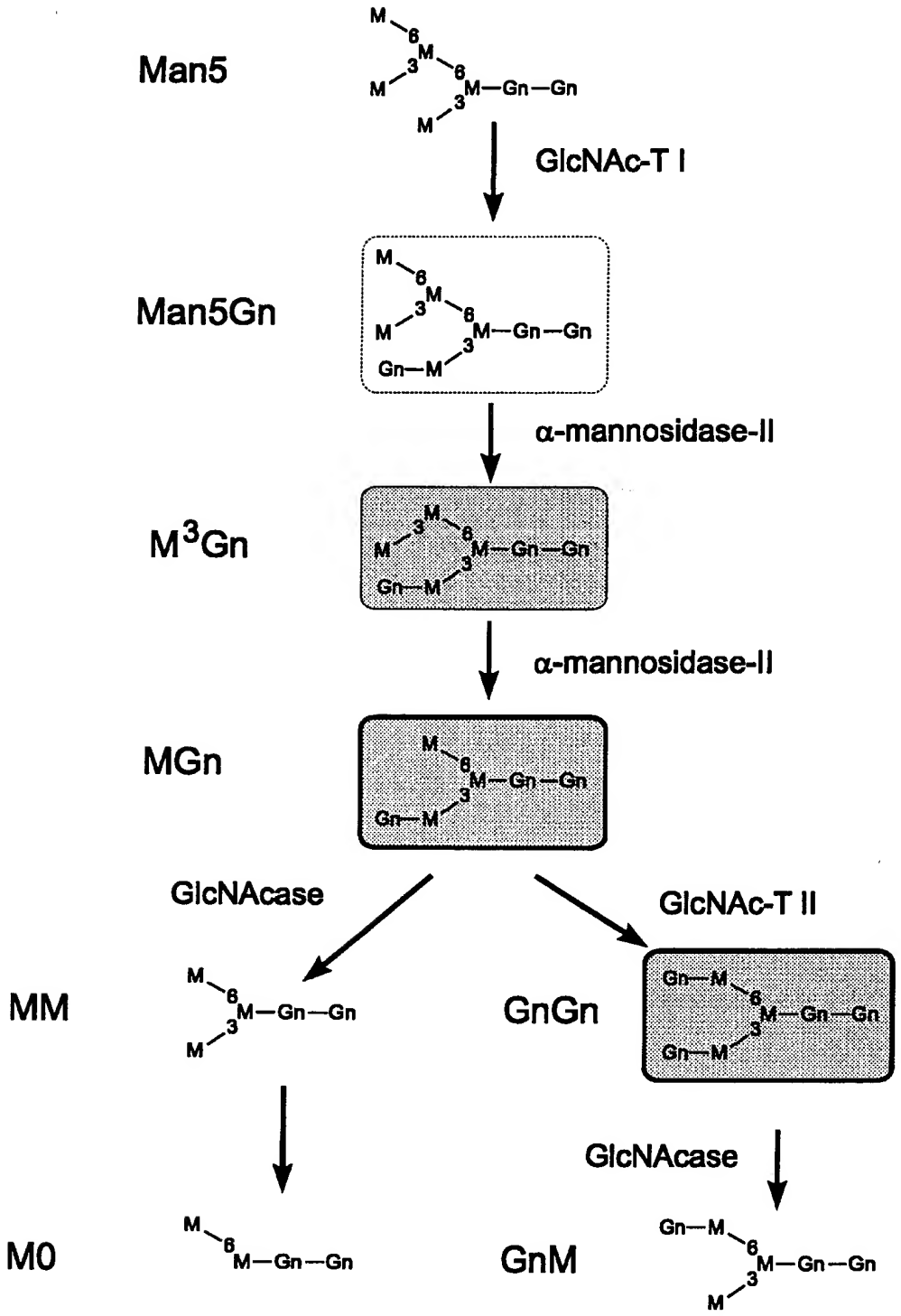

Figure 6. Terminal GlcNAc residues and the biosynthesis of "complex" N-glycans in insect cells. The proposed pathway for the conversion of Man5 to "paucimannosidic" N-glycans is shown under neglect of fucosylation. However, possible substrates for core fucosyltransferases (see Fig. 7) are emphasised. Man5Gn may be assumed to represent a fucosyltransferase substrate but this remains to be shown. While there is evidence that the removal of the GlcNAc residue from the α1,3-arm is caused by a Golgi-associated GlcNAcase and thus represents a true biosynthetic event, nothing is yet known about the mannosidase responsible for the removal of the α1,3-linked mannose.

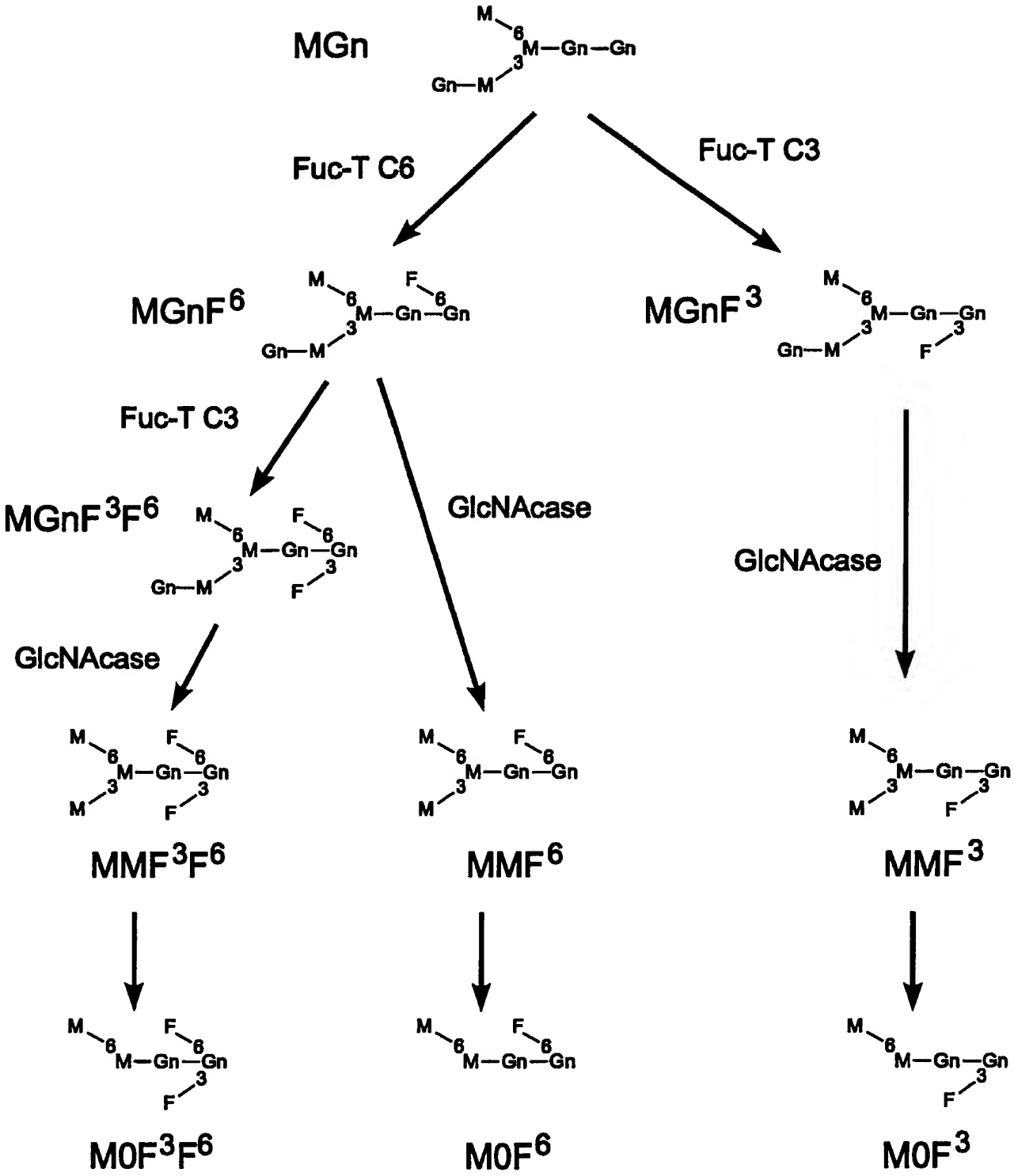

Figure 7. Core fucosylation pathways in insect cells. The figure shows possible sequences of the action of core α1,3- and core α1,6-fucosyltransferase and GlcNAcase. Other transferases are not considered.

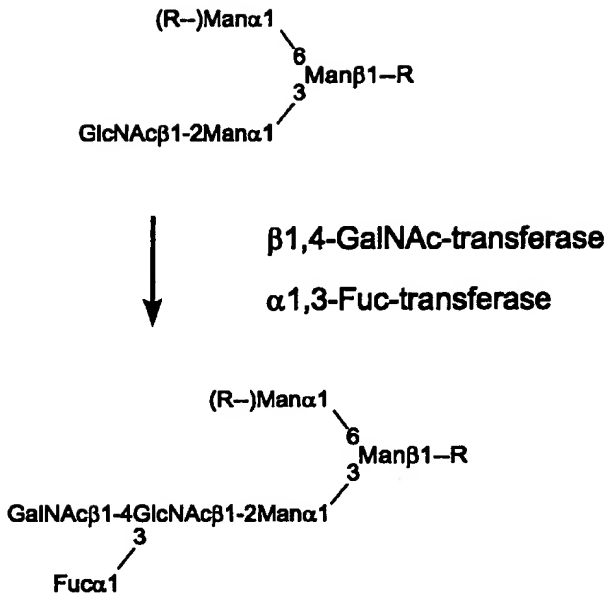

Figure 8. The "LacdiNAc pathway" in insects. Although a GalNAc-transferase has been detected in *T. ni* cells [124], the respective structures have, apart from glycoproteins of non-insect origin [147], hitherto only been found in honeybee venom glycoproteins [101,120].

capacity (see above), the "complex type" N-glycans from insects are very different from their mammalian models. First we shall consider strategies that help to produce mammalian-like glycoproteins in insect cells prior to reviewing the first attempts in this direction.

In order to produce a glycoprotein with "humanised", e.g. sialylated, biantennary N-glycans we have to ensure several things:

(1) core α1,3-fucosylation must not occur, either by the correct choice of cell line or by some form of metabolic engineering;
(2) the GlcNAc residue attached by GlcNAc-transferase I must be retained on the oligosaccharide by minimising GlcNAcase activity (either by choice of cell line, use of an inhibitor, anti-sense RNA or gene knock-out) and by protecting it by further substituents (see below). It appears likely that in most cell lines the endogenous GlcNAc-transferase I activity is sufficient to convert most of the $Man_5GlcNAc_2$ offered [16]. However, this may also be a matter of concern.
(3) the GlcNAc-transferase II activity must be high enough to initiate the second antenna on at least most of the N-glycans. Despite differences among the cell lines studies so far, this generally does not seem to be the case.
(4) sufficient β1,4-Gal-transferase must be present. Even those cell lines which appear to express this activity produce only small amounts of galactosylated N-glycans [104]. Thus a transgenic approach appears to be necessary (see below).
(5) finally there is the issue of sialylation. This might not be just restricted to the expression of sialyltransferase (whichever linkage is required). It is even the mere availability of CMP-sialic acid in the lumen of the Golgi which has to be ensured. Actually, at present we cannot even be certain whether lepidopteran cells synthesise sialic acid at all—not to mention its activation and transport across the Golgi membrane.

Even though it obviously poses an enormous challenge, attempts to "mammalianise" the glycosylation capacity of insect cells have been started. Influenza virus haemagglutinin has been expressed in Sf9 cells in parallel with human GlcNAc-transferase I [102]. Compared to a control where haemagglutinin was expressed in the absence of heterologous GlcNAc-transferase, the amount of terminal GlcNAc-containing N-glycans was increased fourfold. However, two thirds of the N-glycans were still mannose-terminated truncated structures probably as a result of the action of the "processing GlcNAcase" (see above). Certainly, there is not a shortage of GlcNAc-transferase I in Sf9 and other insect cells and the increase in hybrid structures caused by co-expression of this enzyme with haemagglutinin must be regarded as a re-transfer of GlcNAc to already processed N-glycans [16]. The second attempt uses virus mediated or stable expression of β1,4-Gal-transferase [38,39]. This latter and particularly interesting approach was convincingly shown to enable Sf9 cells to galactosylate baculovirus gp64 or human tissue plasminogen activator (tPA) [39]. Regrettably, a detailed structural analysis of N-glycans on these products has not yet been published.

It will be interesting to see whether an insect cell line with multiple stably expressed glycosyltransferases will in fact be stable enough to promote large scale production of recombinant glycoproteins. In the meanwhile, the reader is encouraged to enjoy the beauty of wild type lepidoptera, i.e. butterflies.

Note

During the publishing of this manuscript, a review on stable expression of recombinant proteins [1], a review on the engineering of N-glycosylation pathways [2] and a paper on the effect of inhibitors on N-glycan processing [3] in insect cells have appeared.

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